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Full paper

Rac1 and ROCK are implicated in the cell surface delivery of GLUT4 under the control of the insulin signal mimetic diDCP-LA-PE

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ABSTRACT

The phosphatidylethanolamine derivative 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidylethanolamine (diDCP-LA-PE) promoted GLUT4 translocation to the cell surface in differentiated 3T3-L1-GLUT4myc adipocytes through a pathway along a phosphatidylinositol 3-kinase (PI3K)/3-phosphoinositide-dependent protein kinase-1 (PDK1)/Akt axis, that mimics insulin signaling. Moreover, diDCP-LA-PE-induced GLUT4 translocation was suppressed by inhibitors of the Rho GTPase Rac1 and Rho-associated coiled-coil-containing protein kinase (ROCK) or knocking-down Rac1 and ROCK1. The results of the present study show that Rac1 and ROCK are critical for regulation of GLUT4 trafficking by diDCP-LA-PE as well as insulin.

1. Introduction

Insulin promotes GLUT4-mediated glucose uptake into skeletal muscle cells and adipocytes through a pathway along an insulin receptor (IR)/IR substrate 1 (IRS-1)/PI3K/PDK1/Akt axis (1). Insulin is shown to activate a variety of PKC isozymes including PKCα, -βII, -δ, -λ/ι and -ζ (2–5), and of them PKCλ/ι and -ζ stimulate GLUT4 translocation to the cell surface, regardless of the pathway described above (6–8). In addition, PKCε may also participate in the regulation of GLUT4 trafficking (9). Accumulating evidence has pointed to the implication of Rho GTPases such as RhoA, Rac1, Cdc42 and TC10 in insulin-regulated GLUT4 trafficking (10, 11). The RhoA effector ROCK, alternatively, plays a role in the regulation of insulin-stimulated GLUT4-mediated glucose uptake (12, 13).

We have earlier found that the phosphatidylethanolamine derivative diDCP-LA-PE, which we have originally synthesized, has the potential to inhibit PTP1B and activate a variety of PKC isozymes (14). PTP1B inhibition could enhance an IR/IRS-1/PI3K/PDK1/Akt signaling pathway due to inhibition of tyrosine dephosphorylation of IR and IRS-1. Notably, diDCP-LA-PE activated the novel PKC isozymes PKCζ and PKCλ/ι, although such effect was not obtained with the other phospholipid derivatives 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidyl-L-serine (diDCP-LA-PS), 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidylcholine (diDCP-LA-PC), or 1,2-*O*-bis-[8-{2-(2-pentyl-cyclopropylmethyl)-cyclopropyl}-octanoyl]-*sn*-glycero-3-phosphatidyl-D-1-inositol (diDCP-LA-PI) (14). Then, we postulated that diDCP-LA-PE could mimic intracellular insulin signaling.

To address this hypothesis, the present study investigated diDCP-LA-PE-induced GLUT4 translocation in differentiated 3T3-L1-GLUT4myc adipocytes and the underlying mechanism. We show here that diDCP-LA-PE as well as insulin stimulates GLUT4 translocation to the cell surface through a PI3K/PDK1/Akt pathway and that Rac1 and ROCK also participate in the regulation of the GLUT4 trafficking.

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2. Materials and methods

2.1. Cell culture

3T3-L1-GLUT4myc fibroblast cell line, expressing GLUT4myc that is constructed by inserting a human c-MYC epitope (14 amino acids) into the first ectodomain of GLUT4. Cells were cultured by the method as described previously (1). We have confirmed in the Oil-Red O staining and Western blot analysis using an anti-peroxisome proliferator-activated receptor γ antibody that cells used here are well differentiated into adipocytes.

2.2. Monitoring of GLUT4 trafficking

Differentiated 3T3-L1-GLUT4myc adipocytes were separated into the cytosolic and plasma membrane fractions by the method as previously described (1), and GLUT4 in each fraction was quantified in the Western blot analysis by detecting the exofacial MYC tag using an anti-mouse c-myc antibody (1:10000 dilution) (Merck Millipore, Darmstadt, Germany).

2.3. Construction and transfection of siRNA

The siRNAs to silence the PI3 kinase p85α (PI3K)-, PDK1-, Akt1/2-, Rac1-, or ROCK1-targeted gene and the negative control (NC) siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) or Ambion (Carlsbad, CA, USA). siRNAs (50 pmol/dish) were transfected into differentiated 3T3-L1-GLUT4myc adipocytes using a Lipofectamine reagent (Invitrogen, Carlsbad, CA, USA), and cells were used for experiments 48 h after transfection.

2.4. Inhibitors

Inhibitors used here are genistein (Nacalai Tesque, Kyoto, Japan), wortmannin (Cayman Chemical Company, Ann Arbor, MI, USA), MK2206 (Selleck Chemicals, Houston, TX, USA), BX912 (Axon

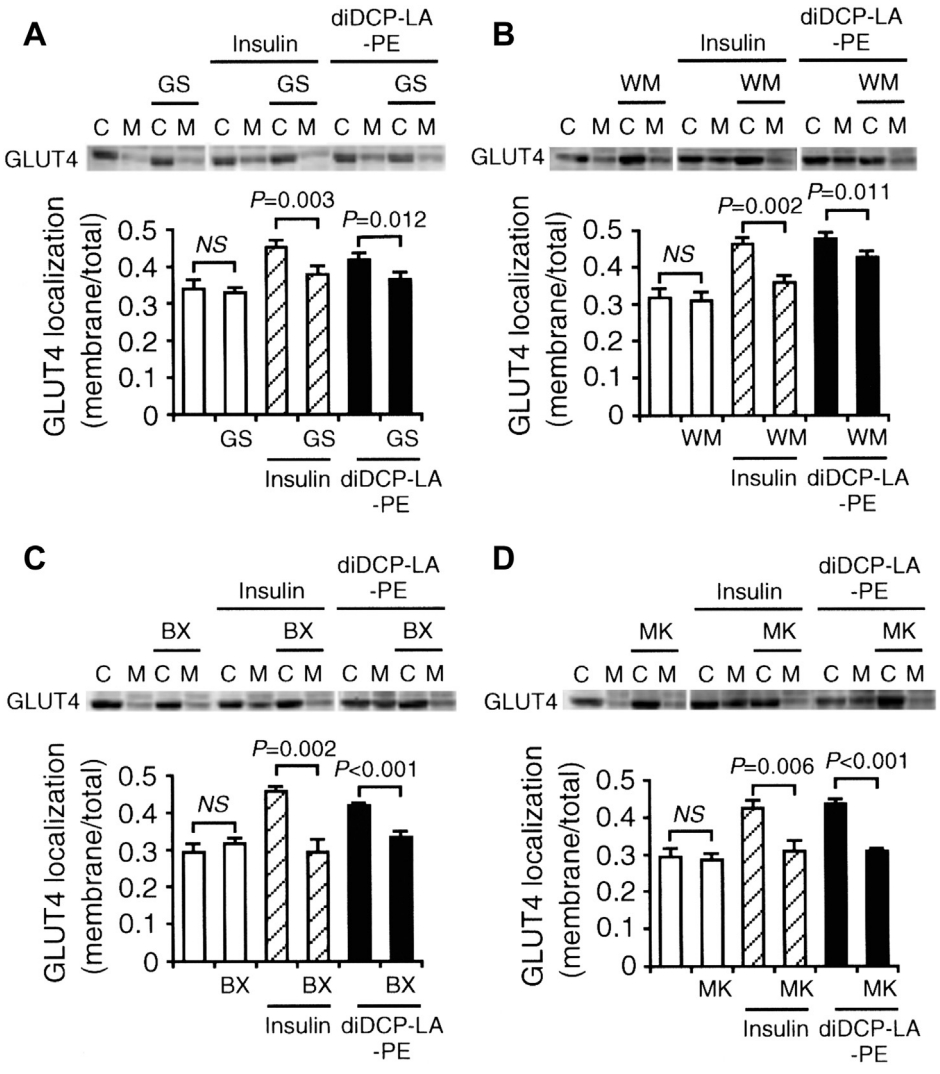

Fig. 1. Insulin and diDCP-LA-PE promotes GLUT4 translocation towards the cell surface through a pathway along an IRS-1/PI3K/PDK1/Akt axis. Differentiated 3T3-L1-GLUT4myc adipocytes treated with insulin (100 nM) or diDCP-LA-PE (1 μM) in the presence and absence of genistein (GS) (50 μM) (**A**), wortmannin (WM) (1 μM) (**B**), BX912 (BX) (100 nM) (**C**) or MK2206 (MK) (5 μM) (**D**) for 20 min. C, cytosol fraction; M, plasma membrane fraction. In the graphs, each column represents the mean (±SEM) signal intensity for GLUT4 on the plasma membrane relative to that for whole cells (n = 4 independent experiments). *P* values, ANOVA followed by a Bonferroni correction. *NS*, not significant.

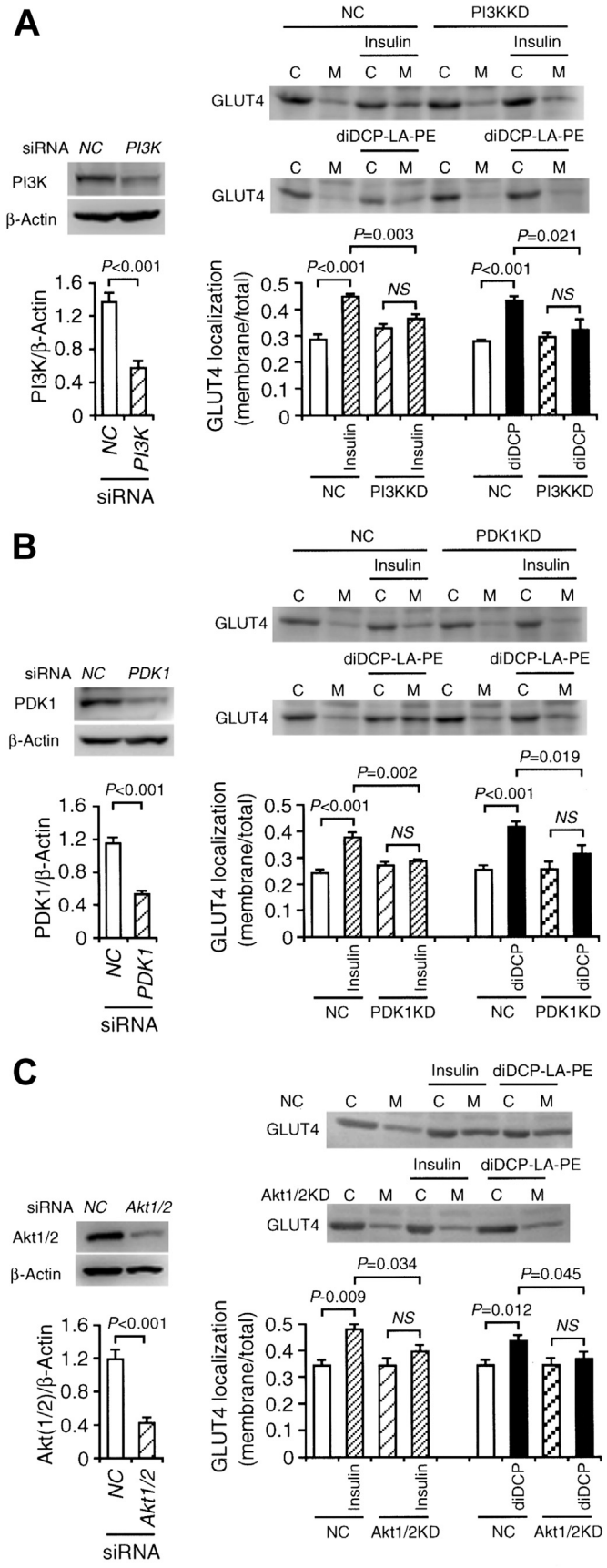

Fig. 2. Insulin- and diDCP-LA-PE-induced GLUT4 translocation is suppressed by knocking-down PI3K, PDK1 or Akt1/2. Differentiated 3T3-L1-GLUT4myc adipocytes, transfected with siRNAs for PI3K (**A**), PDK1 (**B**) or Akt1/2 (**C**), were treated with insulin (100 nM) or diDCP-LA-PE (diDCP) (1 μM) for 20 min. In the graphs in the left panels, each column represents the mean (±SEM) signal intensity for each protein normalized by that for β-actin (n = 4 independent experiments). *P* values, unpaired *t*-test. In the graphs in the right panels, each column represents the mean (±SEM) signal intensity for GLUT4 on the plasma membrane relative to that for whole cells (n = 4 independent experiments). *P* values, ANOVA followed by a Bonferroni correction. *NS*, not significant.

Medchem, Reston, VA, USA), NSC23766 (Tocris Bioscience, Bristol, UK), and Y27632 (Merck Millipore, Darmstadt, Germany).

2.5. Western blotting

Western blotting was carried out using an anti-rabbit PI3K antibody (Sigma, St. Louis, MO, USA), an anti-rabbit PDK1 antibody (Sigma), an anti-rabbit Akt1/2 antibody (Cell Signaling Technology, Inc., Danvers, MA, USA), an anti-rabbit Rac1 antibody (Sigma), an anti-rabbit ROCK1 antibody (Cell Signaling), an anti-mouse β-actin antibody (Sigma), which are diluted at 1/10000, followed by a horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody or anti-rabbit IgG antibody (MP Biomedicals, LLC., Solon, OH, USA).

2.6. diDCP-LA-PE

diDCP-LA-PE was synthesized in our laboratory by the method described previously (14). diDCP-LA-PE was dissolved with a chloroform/dimethyl sulfoxide (DMSO) mixture and diluted with Krebs-Ringer-HEPES buffer (136 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$ and 20 mM HEPES, pH 7.5) containing 0.2% (w/v) bovine serum albumin (BSA) supplemented with 10 mM glucose.

2.7. Statistical analysis

Statistical analysis was carried out using unpaired *t*-test and analysis of variance (ANOVA) followed by a Bonferroni correction.

3. Results

Insulin increased cell surface localization of GLUT4 in differentiated 3T3-L1-GLUT4myc adipocytes (Fig. 1A–D). The effect of insulin was abrogated by the tyrosine kinase inhibitor genistein (GS), the PI3K inhibitor wortmannin (WM), the PDK1 inhibitor BX912 (BX) or the Akt inhibitor MK2206 (MK), although each inhibitor had no effect on cell surface localization of GLUT4 (Fig. 1A–D). This indicates that insulin stimulates GLUT4 translocation towards the cell surface through a pathway along an IR/IRS-1/PI3K/PDK1/Akt axis. Likewise, diDCP-LA-PE increased cell surface localization of GLUT4, and the effect was significantly inhibited by GS, WM, BX or MK (Fig. 1A–D). This suggests that diDCP-LA-PE stimulates GLUT4 translocation towards the cell surface through a pathway shared with insulin signaling.

To obtain further evidence for this notion, PI3K, PDK1 or Akt1/2 was knocked-down using each siRNA. Whether each protein is successfully knocked-down was confirmed by quantifying each protein in the Western blot analysis (Fig. 2A–C). Insulin increased cell surface localization of GLUT4 in cells transfected with the NC siRNA, and the effect was clearly inhibited by knocking-down PI3K, PDK1 or Akt1/2 (Fig. 2A–C). This implies that insulin stimulates GLUT4 delivery to the cell surface in a PI3K-, PDK1- and Akt-dependent manner. diDCP-LA-PE increased cell surface localization of GLUT4 in cells transfected with the NC siRNA, and the effect

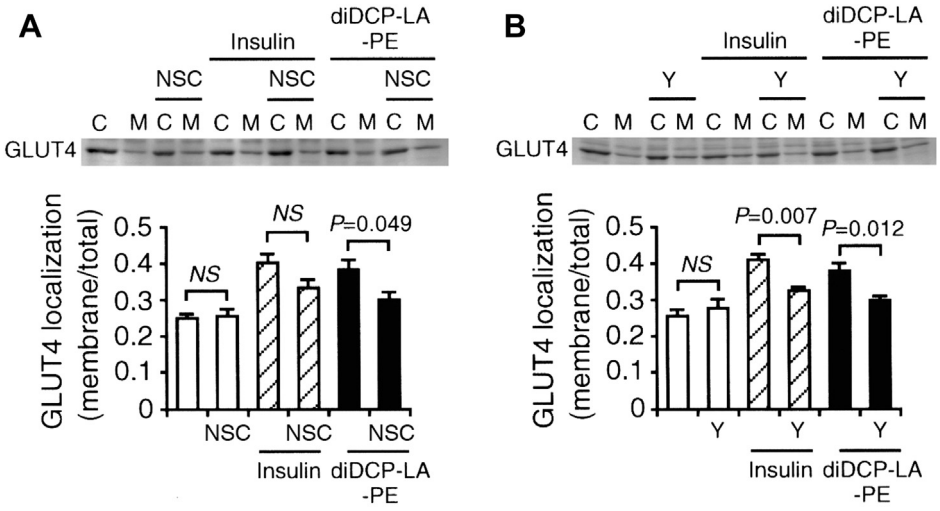

Fig. 3. Insulin and diDCP-LA-PE promotes GLUT4 translocation towards the cell surface by targeting Rac1 or ROCK. Differentiated 3T3-L1-GLUT4myc adipocytes treated with insulin (100 nM) or diDCP-LA-PE (1 μM) in the presence and absence of NSC23766 (NSC) (50 μM) (**A**) or Y27632 (Y) (10 μM) (**B**) for 20 min. In the graphs, each column represents the mean (±SEM) signal intensity for GLUT4 on the plasma membrane relative to that for whole cells (n = 4 independent experiments). *P* values, ANOVA followed by a Bonferroni correction. *NS*, not significant.

was also suppressed by knocking-down PI3K, PDK1 or Akt1/2 (Fig. 2A–C). This accounts for diDCP-LA-PE-induced GLUT4 translocation through a PI3K/PDK1/Akt pathway.

Moreover, insulin-induced increase in the cell surface localization of GLUT4 was inhibited by the Rac1 inhibitor NSC23766 (NSC), but not significantly (Fig. 3A). diDCP-LA-PE-induced GLUT4 translocation was significantly inhibited by NSC (Fig. 3A). Expression of Rac1 in cells transfected with the Rac1 siRNA was significantly decreased as compared with that in cells transfected with the NC siRNA (Fig. 4A), confirming successful knock-down of Rac1. The effects of insulin and diDCP-LA-PE on GLUT4 translocation were significantly prevented by knocking-down Rac1 (Fig. 4A). Collectively, these results indicate that Rac1 is implicated in the regulation of insulin- and diDCP-LA-PE-induced GLUT4 translocation.

Notably, insulin- and diDCP-LA-PE-induced increase in the cell surface localization of GLUT4 was definitely suppressed by the ROCK inhibitor Y27632 (Y) (Fig. 3B). In the Western blot analysis, the signal intensity reactive to an anti-ROCK1 antibody in cells transfected with the ROCK1 siRNA was obviously reduced as compared with that in cells transfected with the NC siRNA (Fig. 4B), indicating successful knock-down of ROCK1. The effects of insulin and diDCP-LA-PE on GLUT4 translocation were significantly prevented by knocking-down ROCK1 (Fig. 4B). Taken together, these results indicate that ROCK is also implicated in the regulation of insulin- and diDCP-LA-PE-induced GLUT4 translocation.

4. Discussion

IR is a receptor tyrosine kinase (RTK), and when activated IR phosphorylates its own receptor and IRS-1 at the tyrosine residue. Phosphorylated IRS-1 recruits and activates PI3K, to produce phosphatidylinositol 3,4,5-triphosphate [PI(3,4,5)P_3] by phosphorylating phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2]. PI(3,4,5)P_3 binds to and activates PDK1. PI3K and PDK1 activate Akt by phosphorylating at the serine and threonine residues, which triggers GLUT4 translocation towards the cell surface (Fig. 5). PI3K also activates PKCζ, which participates in the regulation of GLUT4 translocation (Fig. 5). The phosphatidylethanolamine derivative diDCP-LA-PE serves as a potent PTP1B inhibitor and a PKCζ activator (14). diDCP-LA-PE-induced PTP1B inhibition could enhance IR signaling by preventing tyrosine dephosphorylation of IR and IRS-1. The PTP1B inhibitor sodium orthovanadate, however, did not affect GLUT4 translocation, while it enhanced serine phosphorylation of Akt (unpublished data). This interprets that only PTP1B inhibition can not activate a PI3K/PDK1/Akt pathway sufficiently to translocate GLUT4. diDCP-LA-PE promoted GLUT4 delivery to the plasma membrane. Collectively, these results suggest that PKCζ activation in addition to PTP1B inhibition may be required for GLUT4 translocation. diDCP-LA-PE, thus, appears to stimulate GLUT4 translocation towards the cell surface by the mechanisms relevant to activation of a PI3K/PDK1/Akt pathway in association with PTP1B inhibition and to direct PKCζ activation, in a fashion that mimics insulin signaling.

The most striking finding in the present study is that Rac1 and ROCK are implicated in the regulation of insulin- and diDCP-LA-PE-induced GLUT4 translocation. In the insulin signaling pathway, PI3K activates Rac1 by activating the Rac1 guanine nucleotide exchange factor (GEF) Tiam1 (15) and activated Rac1 promotes GLUT4 translocation towards the cell surface by remodeling actin filaments (10) (Fig. 5). Interestingly, constitutive activation of FLJ00068, a Rac1 GEF, enables GLUT4 translocation without insulin stimulation (16). Taken together, diDCP-LA-PE appears to promote GLUT4 translocation not only through a pathway along a PI3K/PDK1/Akt axis but a pathway along PI3K/Rac1 axis still in the absence of insulin.

Activated Rac1, in turn, binds to and activate the Rac1 effector Dbs, a RhoA GEF (17). Activated RhoA activates the RhoA effector ROCK (18), leading to GLUT4 translocation towards the cell surface and an increase in the glucose uptake into cells (Fig. 5). ROCK includes two isoforms ROCK1 and ROCK2. ROCK1 phosphorylates and inhibits cofilin bearing actin depolymerization, thereby promoting

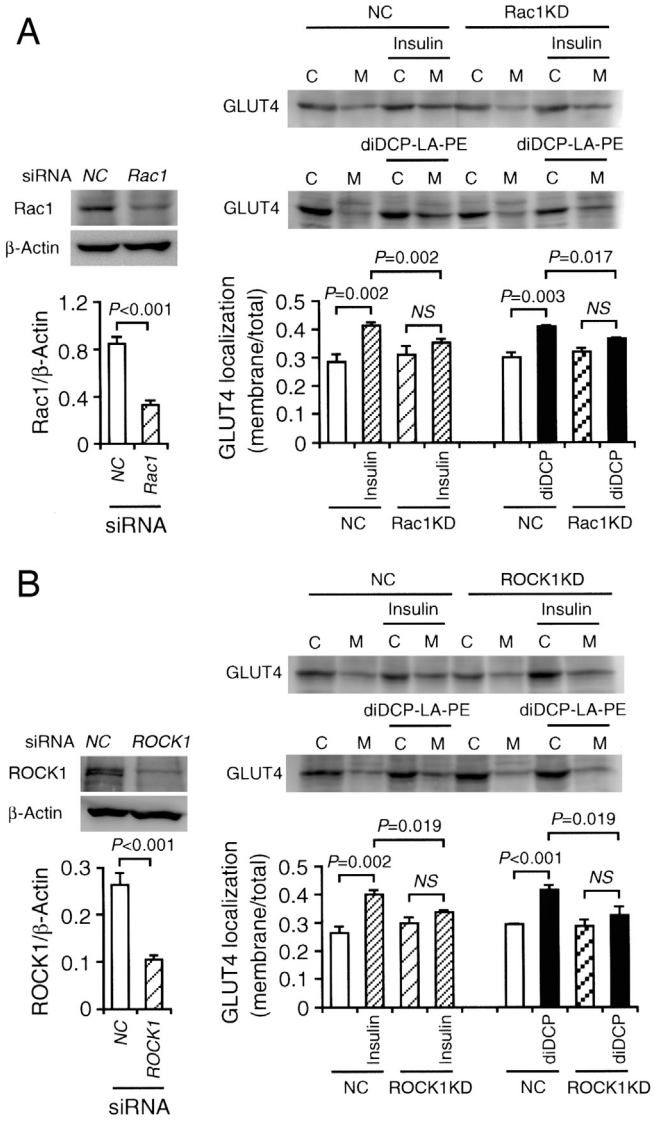

Fig. 4. Insulin- and diDCP-LA-PE-induced GLUT4 translocation is suppressed by knocking-down Rac1 or ROCK1. Differentiated 3T3-L1-GLUT4myc adipocytes, transfected with siRNAs for Rac1 (**A**) or ROCK1(**B**), were treated with insulin (100 nM) or diDCP-LA-PE (diDCP) (1 μM) for 20 min. In the graphs in the left panels, each column represents the mean (±SEM) signal intensity for each protein normalized by that for β-actin (n = 4 independent experiments). *P* values, unpaired *t*-test. In the graphs in the right panels, each column represents the mean (±SEM) signal intensity for GLUT4 on the plasma membrane relative to that for whole cells (n = 4 independent experiments). *P* values, ANOVA followed by a Bonferroni correction. *NS*, not significant.

GLUT4 translocation (13, 19). ROCK2, on the other hand, establishes a positive-feedback loop in the IRS-1/PI3K/PDK1/Akt pathway relevant to GLUT4 translocation by phosphorylating IRS-1 at Ser632/635 to enhance recruitment of PI3K (12). Consequently, ROCK may also be an essential target of diDCP-LA-PE as well as insulin in the regulation of GLUT4 translocation.

In conclusion, the results of the present study demonstrate that Rac1 and ROCK are implicated in the regulation of GLUT4 translocation induced by the phosphatidylethanolamine derivative diDCP-LA-PE mimicking insulin signaling.

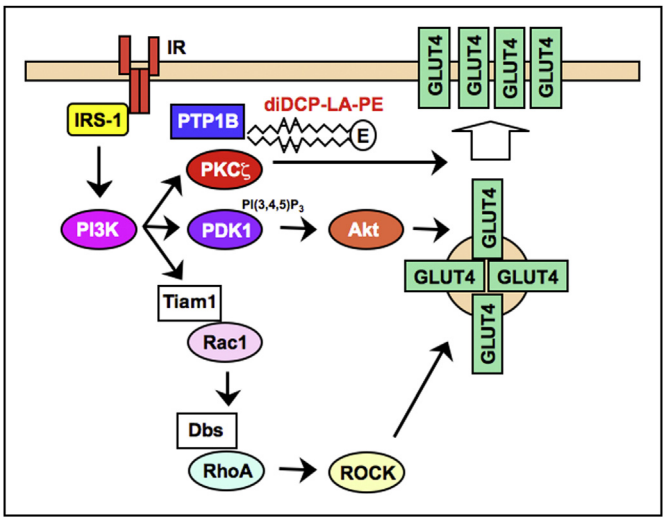

Fig. 5. Schematic regulatory pathways for insulin- and diDCP-LA-PE-induced GLUT4 translocation. Insulin- or diDCP-LA-PE promotes GLUT4 translocation towards the cell surface through a PI3K/PDK1/Akt pathway and by activating PKCζ. PI3K, alternatively, activates the Rac1 GEF Tiam1, to activate Rac1. Activated Rac1, in turn, binds to and activate the Rac1 effector Dbs, a RhoA GEF, activate RhoA. Activated RhoA activates the RhoA effector ROCK, leading to GLUT4 translocation towards the cell surface.

Conflicts of interest

The authors declare that they have no conflicts of interest to disclose.

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References

(1) Tsuchiya A, Kanno T, Nishizaki T. PI3 kinase directly phosphorylates Akt1/2 at Ser473/474 in the insulin signal transduction pathway. J Endocrinol. 2013;220:49–59.
(2) Braiman L, Alt A, Kuroki T, Ohba M, Bak A, Tennenbaum T, et al. Protein kinase Cδ mediates insulin-induced glucose transport in primary cultures of rat skeletal muscle. Mol Endocrinol. 1999;13:2002–2012.
(3) Braiman L, Sheffi-Friedman L, Bak A, Tennenbaum T, Sampson SR. Tyrosine phosphorylation of specific protein kinase C isoenzymes participates in insulin stimulation of glucose transport in primary cultures of rat skeletal muscle. Diabetes. 1999;48:1922–1929.
(4) Rosenzweig T, Braiman L, Bak A, Alt A, Kuroki T, Sampson SR. Differential effects of tumor necrosis factor-alpha on protein kinase C isoforms α and δ mediate inhibition of insulin receptor signaling. Diabetes. 2002;51:1921–1930.
(5) Sampsona SR, Cooper DR. Specific protein kinase C isoforms as transducers and modulators of insulin signaling. Mol Genet Metab. 2006;89:32–47.
(6) Etgen GJ, Valasek KM, Broderick CL, Miller AR. *In vivo* adenoviral delivery of recombinant human protein kinase C-ζ stimulates glucose transport activity in rat skeletal muscle. J Biol Chem. 1999;274:22139–22142.
(7) Farese RV. Function and dysfunction of aPKC isoforms for glucose transport in insulin-sensitive and insulin-resistant states. Am J Physiol Endocrinol Metab. 2002;283:E1–E11.
(8) Farese RV, Sajan MP, Yang H, Li P, Mastorides S, Gower Jr WR, et al. Muscle-specific knockout of PKC-λ impairs glucose transport and induces metabolic and diabetic syndromes. J Clin Invest. 2007;117:2289–2301.

(9) Tsuchiya A, Kanno T, Nishizaki T. Diacylglycerol promotes GLUT4 translocation to the cell surface in a PKCε-dependent and PKCλ/ι and -ζ-independent manner. Life Sci. 2013;93:240–246.
(10) Ishikura S, Koshkina A, Klip A. Small G proteins in insulin action: Rab and Rho families at the crossroads of signal transduction and GLUT4 vesicle traffic. Acta Physiol. 2008;192:61–74.
(11) Satoh T. Rho GTPases in insulin-stimulated glucose uptake. Small GTPases. 2014;5:e28102.
(12) Furukawa N, Ongusaha P, Jahng WJ, Araki K, Choi CS, Kim HJ, et al. Role of Rho-kinase in regulation of insulin action and glucose homeostasis. Cell Metab. 2005;2:119–129.
(13) Chun KH, Araki K, Jee Y, Lee DH, Oh BC, Huang H, et al. Regulation of glucose transport by ROCK1 differs from that of ROCK2 and is controlled by actin polymerization. Endocrinology. 2012;153:1649–1662.
(14) Kanno T, Tsuchiya A, Shimizu T, Nakao S, Tanaka A, Nishizaki T. Effects of newly synthesized DCP-LA-phospholipids on protein kinase C and protein phosphatases. Cell Physiol Biochem. 2013;31:555–564.
(15) Singleton PA, Dudek SM, Chiang ET, Garcia JG. Regulation of sphingosine 1-phosphate-induced endothelial cytoskeletal rearrangement and barrier enhancement by S1P1 receptor, PI3 kinase, Tiam1/Rac1, and α-actinin. FASEB J. 2005;19:1646–1656.
(16) Ueda S, Kataoka T, Satoh T. Activation of the small GTPase Rac1 by a specific guanine-nucleotide-exchange factor suffices to induce glucose uptake into skeletal-muscle cells. Biol Cell. 2008;100:645–657.
(17) Cheng L, Mahon GM, Kostenko EV, Whitehead IP. Pleckstrin homology domain-mediated activation of the rho-specific guanine nucleotide exchange factor Dbs by Rac1. J Biol Chem. 2004;279:12786–12793.
(18) Julian L, Olson MF. Rho-associated coiled-coil containing kinases (ROCK): structure, regulation, and functions. Small GTPases. 2014;5:e29846.
(19) Chiu TT, Patel N, Shaw AE, Bamburg JR, Klip A. Arp2/3- and cofilin-coordinated actin dynamics is required for insulin-mediated GLUT4 translocation to the surface of muscle cells. Mol Biol Cell. 2010;21:3529–3539.